Rsk2 as a modulator in Smn-deficient motoneurons

By

Mr. Sameehan Uday Mahajani

Institute of Clinical Neurobiology

Supervisor - Dr. Michael Sendtner

A project dissertation in partial fulfilment of the requirements of the Qualification phase of Graduate School of Life Sciences at the University of Wuerzburg.

Table of Contents

Abstract

Coffin Lowry Syndrome and Spinal Muscular Atrophy are caused by mutation or loss of Rsk2 and SMN gene respectively. CLS is a rare X-linked syndromic form of severe mental retardation observed in males, whereas SMA is an autosomal recessive disorder and is considered to be one of the leading genetic causes of infant mortality. Smn protein along with its binding partner hnRNP-R aids the transport of β-actin mRNA from the nucleus to the growth cones. β-actin mRNA is essential for axonal elongation and pathfinding. Thus, motoneurons obtained from SMN-knockout mice model are shorter as compared to the wild types. However, Rsk2 is said to be involved in various cellular functions, one of which is to inhibit local translation by activating PDK1. Another paper discussed the evidence that RSKs are capable of phosphorylating Ribosomal protein which is a component of 40S ribosomal subunit required for translation, indicating that RSK signalling contributes to the assembly of translation initiation complex. Thus, this project will deal with the molecular mechanisms linking the two disorders. It would be interesting to see whether double knockout of both Rsk2 and SMN gene has any effect on the elongation of axons obtained from $Rsk2^{-/-}$; $Smn^{-/-}$; $SMN2^{tg/tg}$ mice model.

List of Tables

List of Figures

List of Abbreviations

BDNF - Brain Derived Neurotrophic Factor

CBP - CREB-binding protein

CLS - Coffin Lowry Syndrome

ER-α - Estrogen receptor α

GSK3 - Glycogen Synthase Kinase - 3

LIF - Leukaemia Inhibitory Factor

PDK- 1 - 3-phosphoinositide dependent protein kinase 1

PLD-1 - Phospholipase D-1

RP - Ribosomal protein

SMA - Spinal Muscular Atrophy

SMN - Survival Motor Neuron

snRNP - small nuclear ribonucleoprotein

Introduction

1.1. Coffin-Lowry Syndrome -

Coffin-Lowry syndrome (CLS) is a rare X-linked syndromic form of severe mental retardation observed in males. Females are usually mildly or variably affected *(Hanauer & Young, 2002).* Clinical symptoms include defects in learning, memory formation as well as various skeletal anomalies like prominent forehead, a flat nasal bridge, etc *(Hanauer & Young, 2002; Frodin & Gammeltoft, 1999).* Multipoint linkage analysis done in 1988, confirmed the disease locus to be in the Xp22.2 region *(Hanauer et al, 1988)*. It thus helped in narrowing down the mutated gene in CLS patient to RPS6KA3, which encodes Rsk2 protein belonging to the Rsk ($p90^{rsk}$) family. Other members of the $p90^{rsk}$ family such as Rsk1, Rsk3 and Rsk4 are encoded by 3 different genes. Over 75 different mutations have been identified in 22 exons of RPS6KA3 gene till date. These mutations cause premature termination of Rsk2 protein translation and a truncated, inactive form of Rsk2 is obtained which is degraded quickly *(Dugani et al, 2010).*

1.2. Rsk2 -

Rsk2 is an 82 kDa growth factor activated serine/threonine kinase. The encoded Rsk2 protein is 740 amino acids long. Rsk2 is widely expressed in the nervous system, in brain particularly in the regions important for learning and memory such as the hippocampus. At a cellular level, it is observed in the cytoplasm and the nucleus *(Frodin & Gammeltoft, 1999)*. Though, when stained in motoneurons it was extensively seen in the cytoplasm and the

axonal growth cones. Very little signal was observed in the nucleus of the motoneurons (Fischer et al, 2009).

Rsk2 contains two functional Kinase domains connected with a small linker region. One is the N-terminal region which is responsible for phosphorylating the substrates whereas the C-terminal region is involved in the activation mechanism of Rsk2. There is an autoinhibitory alpha helix region (697-712) present in the C-terminal domain *(Frodin & Gammeltoft, 1999; Poteet-Smith et al, 1999).*

Rsk2 can be activated either by growth factors, hormones or neurotransmitters via the MAPK signalling pathway. All members of $p90^{rsk}$ family are found to be downstream of ERK-MAPK pathway. It has been observed that ERK phosphorylates Thr577 position on the C-terminal of Rsk2 and Ser369 position on the linker region. Once activated, the C-terminal region then autophosphorylates Ser386 position in the linker region. This Ser386 upon phosphorylation generates a docking site that recruits 3-phosphoinositide dependent protein kinase 1 (PDK-1) and increases the activity by 5 fold, thus phosphorylating the N-terminal domain at Ser227 in the activation loop. Phospho-Ser386 after dissociation from PDK-1, binds to a phosphate binding site in the N-terminal domain. This structure co-operates with phosphor-Ser227 in the activation loop to stabilize N-terminal domain in an active conformation, resulting in the stimulation of kinase activity *(Anjum & Blenis, 2008).*

Rsk2 phosphorylates many substrates which are responsible for proliferation, differentiation, survival and normal functioning of the cell. Transcription factors like CREB, c-Fos, Estrogen receptor α (ER-α) and transcriptional co-activator proteins like CREB-binding protein (CBP) and P300 are among the substrates of Rsk2. It is also been said to

phosphorylate Glycogen Synthase Kinase - 3 (GSK3) which regulates mRNA transcription, metabolism, cell fate and differentiation *(Frodin & Gammeltoft, 1999)*.

According to the studies done by Zeniou et al in 2002, Rsk1 and Rsk3 should be able to compensate for the absence of the Rsk2 protein in most tissues, as they are expressed at higher levels than Rsk2. But, in the pyramidal cells of the hippocampus, in the Purkinje cells of the cerebellum and in deep layers of the neocortex of the adult mouse brain, the Rsk2 gene shows very high levels of expression, whereas Rsk1 and Rsk3 mRNA expressions are not detectable. It was also observed that expression of at least Rsk1 or Rsk3 were not increased in response to Rsk2 deficiency. These data support the hypothesis that in these areas Rsk1 and Rsk3 might not be able to fully compensate for RSK2 deficiency in $Rsk2^{-/-}$ mice and in CLS patients leading to the phenotypes observed *(Zeniou et al, 2002).*

Another important paper demonstrates that Rsk2 is a critical regulator of Phospholipase D-1 (PLD-1). As it was known that PLD-1 is essential for fast release of neurotransmitter, the absence of Rsk2 in CLS patients could help in understanding the symptoms like mental retardation. Although, this questions the upstream signalling pathway which is usually required to activate Rsk2. It was suggested that rise in cytosolic calcium influx due to membrane depolarisation was sufficient to stimulate activation of Rsk2 *(Zeniou-Meyer et al, 2008)*.

1.3. Generation of Rsk2 KO mice -

Yang et al in 2004 generated these Rsk2 knockout mice which would be used in the project. They introduced a neomycin cassette along with three stop codons and two LoxP sites on

either side on the cassette. This construct was inserted in Exon 2 of the Rsk2 sequence such that 2Kb and 9Kb sequence was left on the 5' and 3' end respectively. This construct was electroporated into 129SvJ embryonic stem cells and then positive cell clones were injected in C57Bl/6 blastocytes. The cassette was removed by crossing mutant with CMV-Cre transgenic mice *(Yang et al, 2004).*

Mice with disrupted Rsk2 gene show impaired learning ability along with mild motor defects. Whereas in Drosophila, even though it has just one Rsk gene, it is highly homologous to the human Rsk2 isoform. Similar alternations in learning and memory which are seen in CLS patients are observed in mice and drosophila that lack Rsk2 gene *(Fischer et al, 2009)*.

1.4. Plasmids generated by Poteet-Smith et al -

In 1999, Poteet-Smith et al; designed certain plasmids expressing Rsk2. These included a Rsk2 Wild Type expressing vector (Rsk2-wt), a kinase dead Rsk2 expressing vector in which Tyrosine was changed to Alanine in 451 position (Rsk2-K451A) mutating the ATP binding site, a plasmid which consist of 54 amino acid deletion that removes autoinhibitory alpha helix region located on the C-terminal domain of Rsk2 (Rsk2-Δα) and one containing a point mutation in the same region where Lysine is replaced by Alanine in 707 position (Rsk2-Y707A).

Rsk2-Δα and Rsk2-Y707A plasmids are considered to produce constitutively active form of Rsk2 *(Fischer et al, 2009; Poteet-Smith et al, 1999).* When BHK cells where transfected with these two plasmids in the presence of PD98059, which specific inhibits the activation of MEK and thereby reducing phosphorylation of further downstream components, it was

observed that there was phosphorylation of Ser386 (present in the linker region) whereas no bands were recorded when the cells where transfected with Rsk2-wt and Rsk2-K451A plasmid along with the inhibitor PD98059. A phospho-specific antibody was used to examine the phosphorylation of Ser-386 *(Poteet-Smith et al, 1999).* Moreover, Rsk2-Δα lacks the ERK docking site on the C-terminal domain of Rsk2.

Therefore, it is understood that relief of autoinhibition of C-terminal domain is sufficient to increase the activity of N-terminal domain even if ERK phosphorylation is absent *(Poteet-Smith et al, 1999).*

1.5. Spinal Muscular Atrophy -

Spinal Muscular Atrophy (SMA) is a neurodegenerative disease for which there is no effective treatment available although it was first described by Guido Werdnig and Johann Hoffman in 1890s *(Sumner C, 2006)*. SMA is an autosomal recessive disorder and is considered to be one of the leading genetic causes of infant mortality. SMA is caused by the degeneration of α – motoneurons in the spinal cord and the brain stem leading to general physical weakness and death mainly due to respiratory failure *(Lorson et al, 2010)*.

Denervation in SMA causes muscle atrophy predominantly in the proximal muscles i.e. the lower limbs affected more than the upper limbs which are more affected than the intercostal muscles. Facial muscles and diaphragm are generally spared in SMA affected individuals *(Bosboom et al, 2009).* SMA affects the motor system from the anterior horn cell to the muscle and thus, the patient has intact sensory and cognitive functions *(Montes et al,*

2009). SMA is further categorised into types depending upon the severity of the disorder (Table 1)

Type of SMA	**Age of Onset**	**Maximum function achieved**	**Natural age of Death**
Type I	0-6 months	Cannot sit unsupported, non invasive respiratory support required	Less than 2 years
Type II	7-18 months	Can sit unsupported, but require braces to walk	More than 2 years
Type III a	18 months - 3 years	Can walk around independently without any braces, but muscles weakness present	Adult
Type III b	>3 years	Same as Type III a	Adult
Type IV	20-30 years	Can perform all functions unsupported, but mild weakness present	Adult

Table 1 - Classification of Spinal Muscular Atrophy.

The genetic alteration causing SMA was discovered in 1995 when the gene responsible was identified and characterised. The critical region for SMA was found to be 5q11.2-13.3 by linkage analysis. After genetic mapping of the region, it was seen that Survival Motor Neuron (*SMN*) gene *(Lefebvre et al, 1995).*

1.6. SMN -

SMN gene is present in two copies in humans namely, *SMN1* (telomeric copy) and *SMN2* (centromeric copy). Both *SMN1* and *SMN2* genes have nine exons and eight introns with their sequences differing in 5 nucleotides *(Lunn and Wang, 2008).* It has been observed that approximately 94% of the SMA patients lack exon 7 of *SMN1* gene. Other 6% of them show small mutations in exon 7. Exon 7 consists of functionally critical sequence with the stop

codon present near the end *(Ogino and Wilson, 2002).* In 1999, 24 SMA patients were shown to lack previously recorded mutations in *SMN1* gene and were thus considered to have *SMN1*-unrelated SMA *(Wirth et al, 1999).*

Even though mutation or loss of *SMN1* gene gives rise to SMA, it is observed that deletion in *SMN2* gene do not lead to any clinical phenotype *(Hahnen et al, 1996). SMN2* gene is only present in humans, whereas it is absent in mice and even chimpanzees. Zero to three copies of *SMN2* gene is usually present in humans. In a very rare case, a patient suffering from SMA type IV was found to have 8 copies of *SMN2* gene *(Vitali et al, 1999)*. Although *SMN2* gene does not encode enough protein to compensate the loss of *SM dN1* gene in patients, it does help a bit in controlling the severity of the phenotype. The *SMN2* transcript undergoes alternative splicing with skipping the exon 7. This mRNA is therefore translated into a C-terminal alternative SMN protein (SMNΔ7) protein which is rapidly degraded. Only 10% of *SMN2* pre-mRNA is properly spliced and full length SMN protein is encoded. Low SMN protein in SMA patients supports embryonic development, but is found to be inadequate for the survival of motoneurons later *(Lunn and Wang, 2008).*

SMN is a 38 kDa protein and is localised in the nucleus as well as in the cytoplasm. It is ubiquitously expressed but high level was observed in motoneurons of the spinal cord. SMN protein is mainly involved in small nuclear ribonucleoprotein (snRNP) assembly formation. Some of the snRNPs are essential for recognition of splice sites and removal of introns from pre-mRNA. snRNP consist of SMN complex and heptameric ring of Sm proteins. SMN complex, in turn, consist of SMN protein, UNR-interacting protein (UNRIP or STRAP) and GEMIN 2-8 proteins and its function is ATP dependent *(Burghes and Beattie, 2009).*

It has also been reported that SMN protein along with its RNA-binding partner hn RNP-R plays an important role in aiding the transport of β-actin mRNA through the axons of motoneurons *(Glinka et al, 2010)*. Reduced localization of β-actin was observed in the growth cones of $Smn^{-/-}$; SMN2 motoneurons as compared with those of the wild type motoneurons when stained with anti-β-actin antibody *(Rossoll et al, 2007)*. This localisation seems to be essential for axon initiation, growth, guidance as well as branching of the motoneurons *(Rossoll et al, 2003)*.

1.7. Literature -

Fischer et al, 2009 demonstrated that Rsk2 negatively regulates the axonal length in motoneurons. The motoneurons cultured from $Rsk2^{-/-}$ mice were longer in length when compared with Wild type motoneurons. There was no effect of absence of Rsk2 on the motoneuron survival when neurotrophic factors were provided as compared to the wild type motoneurons. Surprisingly, increased survival of $Rsk2^{-/-}$ motoneurons was observed as compared to wild type motoneurons when no neurotrophic factors were added. *(Fischer et al, 2009).*

Another paper published by Dugani et al, 2010 showed that when Rsk2 is absent, there is decreased level of neurogenesis in cultured cortical precursor cells. Knockdown of Rsk2 was performed using Sh-RNA technology which was capable of only 60-80% knockdown. They stated that precursor cells tend to remain undifferentiated rather than differentiating into neurons in absence of Rsk2. Moreover, they show that Rsk2 is not essential for the survival of precursor cells. No such effect was seen in their differentiating into astrocytes in presence or absence of Rsk2 *(Dugani et al, 2010).*

Materials and Methods

2.1. Embryonic motoneuron cultures -

Spinal motoneurons were isolated from lumbar spinal cord of 14 day old mouse embryos. The lumbar spinal cord was dissected, trypsinized with 0.1% trypsin for 15mins at 37^{o}C. Then the motoneurons were enriched using an antibody against the P75^{NTR} receptor and plated at a density of 2000 cells per coverslip. The coverslips were coated with PORN and laminin. Cells were grown in neurobasal media (GIBCO) containing 2% heat inactivated horse serum, 2% B27, 500μM glutamax and BDNF (depending on the experiment). Medium was replaced after 24h and then every second day. The cultures were maintained at 37^{o}C with 5% CO_2.

2.2. Survival Assay -

The survival of the motoneurons was also assessed by counting them once at day 0 (5 hours after plating them), on day 3, on day 5 and on day 7. Specifically, this was done by dividing the 35mm dish with 10mm rings tissue culture plates into halves by drawing a line under the ring and counting the number of half field motoneurons along the line using a 10x microscope objective.

2.3. Immunocytochemistry

Immunocytochemistry is a technique that allows the visualisation of a protein taking advantage of the binding between antigen and antibody. In particular, a first antibody (primary) is used to label the protein of interest, whilst a further antibody (secondary) is usually fluorophore-labelled and recognises the primary antibody (Marotta et al, 2016;

Pietro et al, 2015).

2.4. Staining of Motoneurons -

The isolated motoneurons were grown for 5 to 7 days on laminin coated coverslips. Cells were fixed using freshly-prepared 4% PFA for 15 mins at room temperature and then washed three times with PBS. Each wash lasting for 15 mins. The fixed cells were blocked and permeabilized with blocking buffer containing, Goat serum, Triton X-100 diluted in TBST. It was left at room temperature for one hour. These cells were then incubated at 4^{o}C overnight with primary antibody diluted in blocking buffer. On the next day, cells were washed three times with TBST each for 15mins, before they were subjected to the secondary antibody for 1 hour at room temperature (Mahajani et al, 2010; Wakhloo et al, 2013). The cells were then washed thrice with TBST for 15 mins each and mounted on a glass slide with Mowiol. The stained coverslips were kept in the dark at 4^{o}C for an hour and then visualised under the confocal microscope Leica TCS SP2. The list of primary and secondary antibodies is listed in (table 2)

Antibody	**Company**	**Dilution**
m-MAP2	Sigma	IC : 1:200
Rb-TAU	Sigma	IC: 1:1000
m-Tuj1	Neuromics antibodies	IC: 1:5000
Rb-GFAP	Chemicon international	IC: 1:200
Rb-Rsk2	MBL international	WB: 1: 1000

Rb-GFP	Santa Cruz	WB: 1:5000
Gt α-mCy2	Jackson Immuno Research	IC : 1:400
Gt α-rbCy3	Jackson Immuno Research	IC: 1:400
Gt α-rbHRP	Jackson Immuno Research	WB: 1:5000

Table 2 - List of antibodies used in the experiments

2.5. Axonal length measurement -

Axonal length measurement was done with the help of Image-J software. The longest process of each motoneuron was considered as the axon, but when there were further ramifications, the length of the longest branch was measured. In particular, segmented lines were used to track the axons and then measured.

2.6. Western Blotting

Firstly, BCA assay (kit obtained from Pierce) was carried out to estimate the concentration of protein present in each sample. The reagents were added and the samples were kept at 37^{o}C for 30 mins. Then absorbance was measured at 562nm using a plate reader and the concentrations of proteins in the samples were assessed by comparison with the standard curve.

Western blotting is a technique that allows determining the relative amount of specific proteins namely, the expression of the protein of interest is compared to the one whose expression is not affected by experimental procedure. In particular, certain amount (μg) of protein samples (determined by BCA assay) were diluted 1:3 in 5x loading buffer (2 ml

mercaptoethanol, 20 ml glycerol, 4g of SDS, 25ml of 4x Tris-HCl, 1mg bromphenol blue in

dH_2O) and denatured at 95°C for 5 minutes. Then the samples and a protein ladder (Bio-rad) were loaded on a 10% SDS-polyacrylamide gel and an electric field was applied to achieve protein separation on the basis of their molecular weight. The proteins were next transferred onto a methanol-preactivated nitrocellulose membrane (Hartenstein) by applying an electric field at 250mA for 75 minutes on ice. In order to assess the quality of the transfer, the membranes were stained with Ponceau dye for 5 minutes followed by thorough washes to visualise all the proteins. The membrane was incubated in 5% non fat milk in TBST (40.03g of NaCl, 15.76 g of Tris base, 5ml of Tween (Sigma) in 5 litres of dH_20) at room temperature for 1 hour to prevent non-specific binding of the antibodies. Primary antibodies (Rb-anti-Rsk2 and rb-anti-GFP) were diluted in 1% milk/TBST and incubated overnight at 4°C. The membranes were washed three times with TBST at room temperature, the first wash lasting 15 minutes while other two lasting 5 minutes each. Then, secondary antibodies (anti-rabbit HRP), diluted in 1% milk/TBST were incubated at room temperature for 1 hour followed by three washes with TBST as described above. The proteins of interest were detected using ECL plus Chemiluminescence detection kit (Healthcare – Amersham) and finally the membranes were exposed to X-ray films.

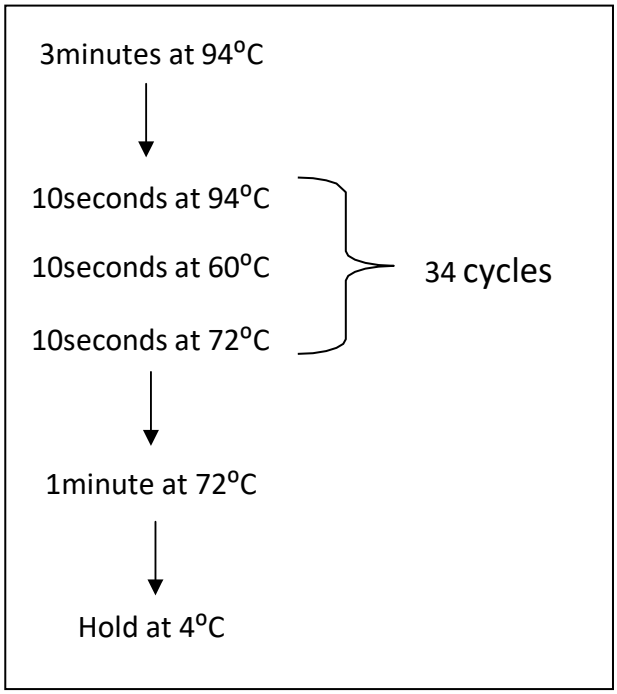
2.7. Genotyping -

Heads were usually collected in DNA lysis buffer for genotyping. They were digested with the help of Proteinase K at 60^oC for 2 hours on a shaker. DNA purification was done by phenol chloroform method. Then the master mix for the PCR reaction was made using the primers, Taq polymerase, etc. The sequence of the primers are given below (Table3)

WL 112	TTG TTG GTT TAC TTT CTT TCG GTC TG
WL 113	AAG ATG ATT GCT TTG CTT AGT TTA

Table 3 - Primers used for genotyping Rsk2 mouse

The PCR cycle condition was as follows -



2.8. Embryonic neuronal stem cell culture -

Dorsal forebrain was extracted from E12 mice embryo brains. They were placed in 100µl of Neurobasal media. They were later trypsinized with 0.1% trypsin for 15 mins at 37°C. They were triturated with P200 pipette, making sure not to form air bubbles and then seeded into flask with 5ml Neurobasal full media containing Glutamax, Pen-Strep, B27, Non-essential amino acids, EGF and bFGF. They were incubated at 37°C with 5% CO_2. The

neurospheres which were seen after two days were harvested and seeded into fresh media at the split ratio 1:3-1:6. For differentiation, these cells were plated on laminin (for neurons) and uncoated plates for astrocytes. Leukaemia Inhibition Factor (LIF) was added to the astrocyte culture to allow differentiation of precursor cells into astrocytes. Neurons were seen after two days in culture, whereas astrocytes were seen in 6 days. The cultures were maintained at 37^{o}C and 5%CO_2 all the time (Wakhloo et al, 2020; Pan et al, 2019).

2.9. Lentiviral vector production -

2.9.1. Splitting the HEK293T cells -

The media was sucked off completely from the flask and washed with T/E once. Prewarmed DMEM media + 10%FCS + Geniticin were added to stop the trypsinization. The cells were triturated and centrifuged at 2800 rpm for 5 mins. Then DMEM medium was added to the pellet and the cells were split according to the density of the culture. The flasks were stored at 37^{o}C and 5% CO_2.

2.9.2. Transfection procedure -

OPTI-MEM (without FCS) & OPTI-MEM (with FCS) was pre-warmed in the incubator at least 3 hours before starting. DNA mix was prepared as following -

OPTI-MEM (without FCS) - 3ml

Packaging vector - 15ug

Pseudotyping vector - 10ug (Total - 31 ug)

Expression plasmid - 6ug

Lipofactamine mix was prepared as following -

OPTI-MEM (without FCS) - 3ml

(Total DNA mix) X 2.3 - 71ug of Lipofactamine

DNA mix was left at room temperature for 5-10 mins and then lipofactamine mix was added to the DNA mix. It was stored at room temperature for 30mins. The flasks containing HEK cells were trypsinized and centrifuged. The transfection mix was added to the cells carefully and mixed. It was left at room temperature for 10 mins. Then, 10 ml of this mix was added to each plate and stored at 37^{o}C and 5% CO_2 for 10-16 hours. Media was changed the next day.

2.9.3. Harvesting of the virus -

Ultracentrifuge tubes and caps were disinfected with ethanol. The media from the plates was sucked off and centrifuged at 3600 rpm for 15 mins. The supernatant was filtered through 0.45µm filter. The filtered media was added to the ultracentrifuge tube first and then 4 ml sucrose was added to the base of the tube without disturbing the media. The tubes were then centrifuged at 25,000 rpm for 2 hours at 4^{o}C. The layer joining the media and sucrose solution was sucked off first and then the entire media. The pellet was kept on Ice and 200µl of pre-cooled TBS-5 buffer was added. The tubes were closed and kept in the ice box in the fridge for 3 hours. The pellet was later resuspended and aliquots of 10 µl each were made and stored at -80^{o}C.

Results

3.1. Motoneuron Culture -

Spinal cords were isolated from E14 CD-1 mice for motoneuron culture. Approximately 2000 motoneurons were plated on each coverslip. Some motoneurons were grown in medium containing BDNF whereas others were grown in medium lacking BDNF (Colombi et al, 2013). Survival assay was performed (Fig1-A). Counting of motoneurons was done on Day 0, day 3, day 5 and day 7.

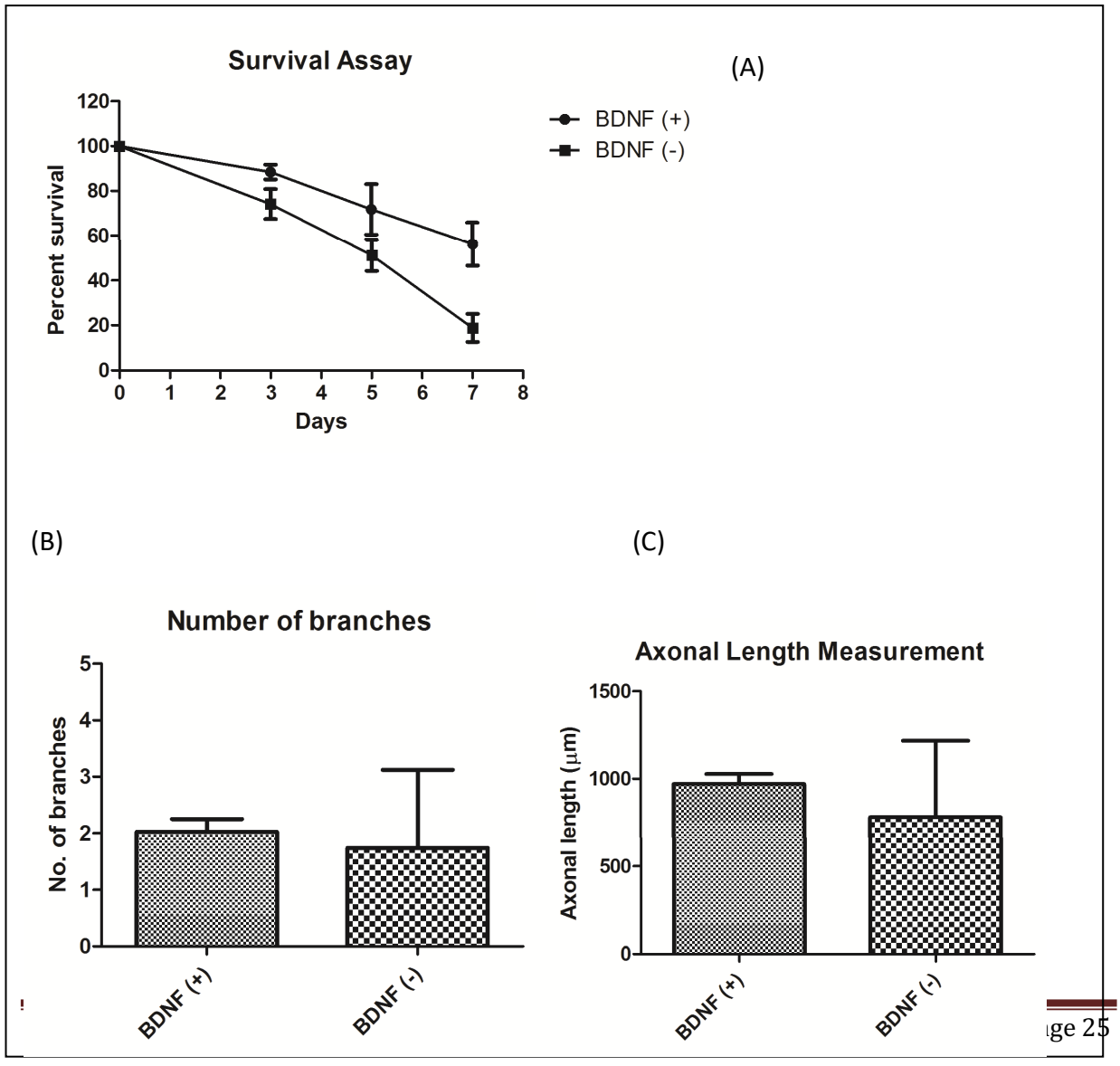


Fig1 - (A) Survival of motoneurons isolated from CD-1 E14 mouse and grown in BDNF +/- medium for 7 days. (B) Number of axonal branches observed after staining the motoneurons (n=35 for each condition). (C) Axonal length measurement done using Image-J software for BDNF +/- motoneurons (n=35 for each condition).

After maintaining the motoneurons in culture for 7 days, they were fixed using PFA and stained with MAP2 (host-Mouse) and TAU (host-Rabbit) antibodies (Fig2). Along with survival assay, number of axonal branches (Fig1-B) and axonal length measurement (Fig1-C) was also done (Giacomini et al, 2016; Mahajani et al, 2017).

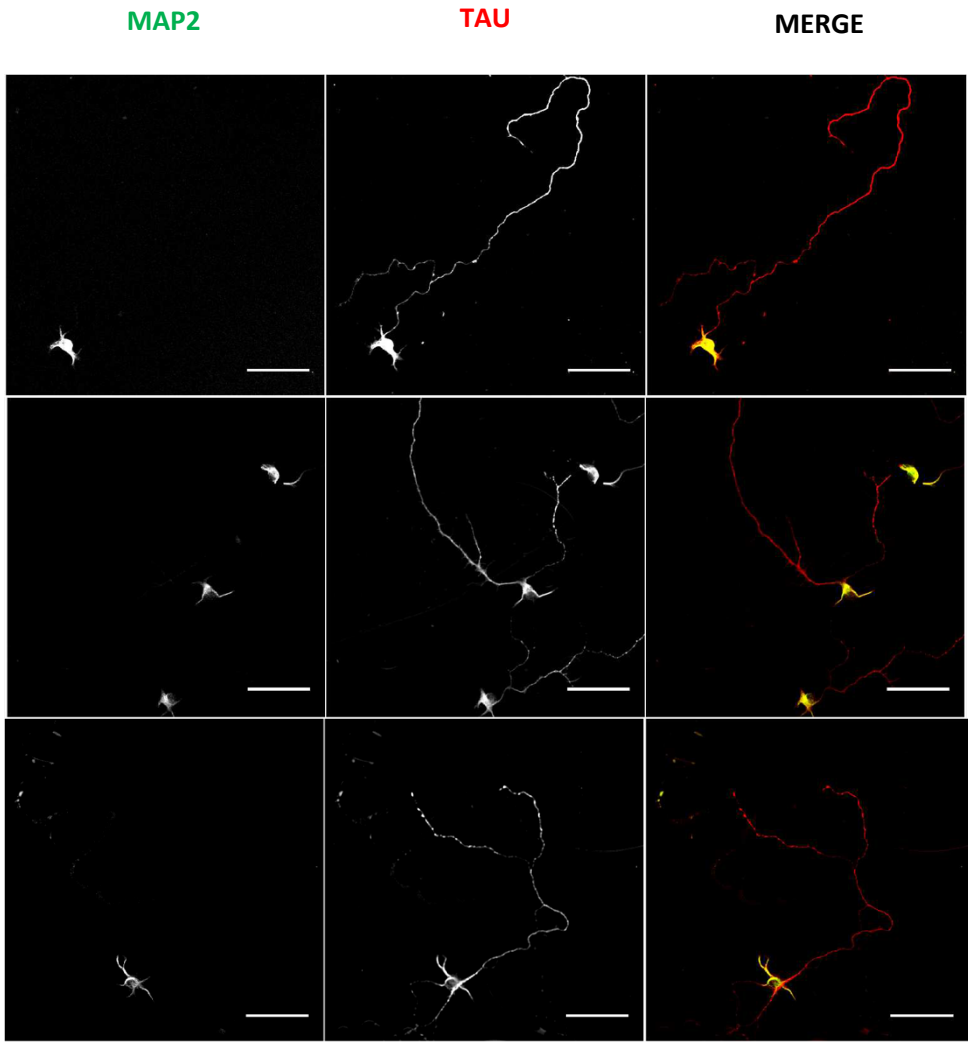


Fig2 - Immunocytochemistry in motoneurons showing dendrites stained using MAP2 antibody and the entire motoneuron stained with TAU. These images were taken after culturing motoneurons for 7 days. The third column shows the merged image of the first two columns. (Scale bar = 50µm)

3.2. Neuronal stem cell culture -

Dorsal forebrain from E12 CD-1 mouse was extracted and seeded in Neurobasal medium with EGF, FGF, non-essential amino acids and pen-strep. Neurospheres were observed after two days in culture (Fig3-A). After another two days, the cells were plated on laminin coated dishes for its differentiation into neurons. Neurons could be seen in the medium in two days

after plating (Fig3-B). Leukaemia Inhibitory Factor (LIF) was added to the medium the next day of plating, for differentiating the neural precursor cells into astrocytes.

(A)

(B)

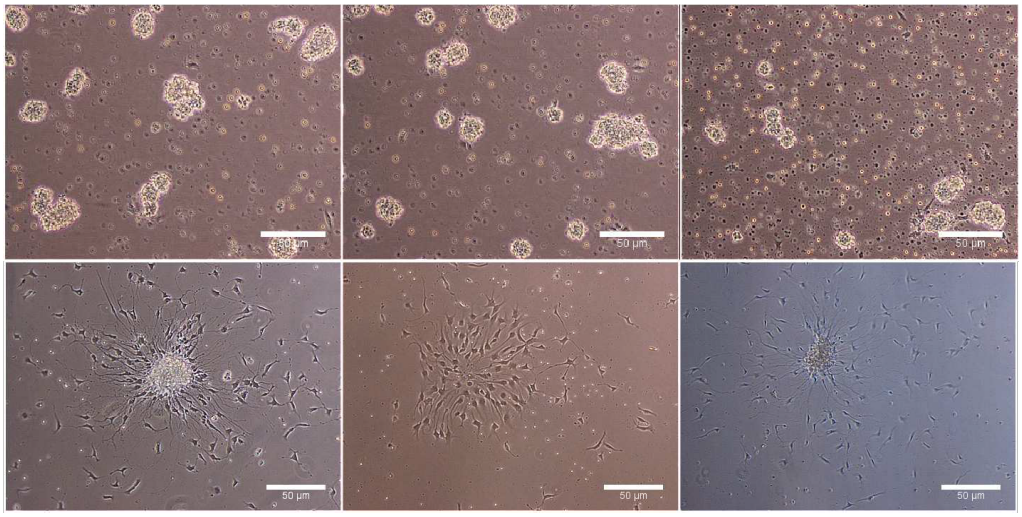
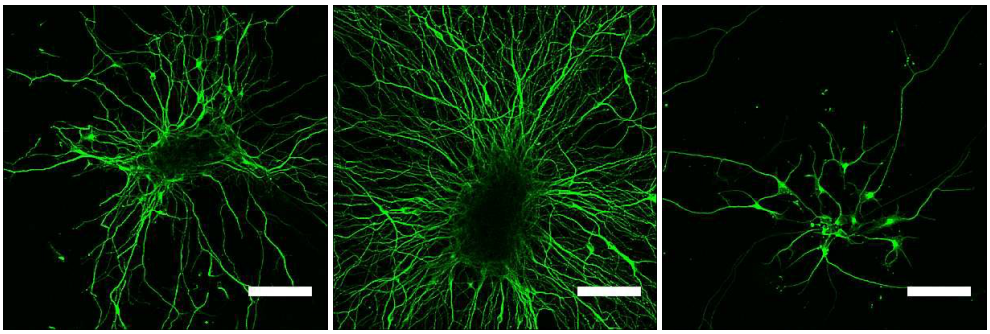


Fig3- (A) Neurospheres seen after two days in culture (B) Precursor cells seen differentiating into neurons within two days after plating them on laminin (Scale=100µm).

Neurons specific staining was done using Tuj1 (host-Rabbit) antibody which recognises the Tubulin β-III present in the motoneurons (Fig4-A). This staining was done by fixing the neurons two days after plating (Mahajani et al, 2019; Mahajani et al, 2021). Astrocytes were detected by GFAP (host-Rabbit) antibody, 6 days after plating (Fig4-B).

(A)



(B)

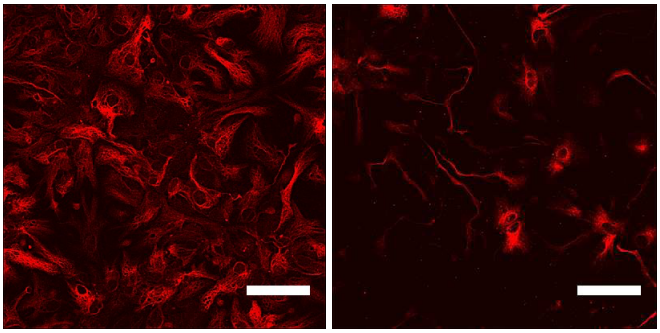


Fig4-(A) Tuj1 staining specific for neurons. It was performed 2 days after plating them on laminin. (B) GFAP staining specific for astrocytes was performed 6 days after plating them on uncoated coverslips (Scale bar= 50µm)

3.3. Plasmid purification -

Four different plasmid vectors were obtained from Dr. Fischer which would be used in this project. The first one was Rsk2 wild type expressing plasmid, second been kinase dead (K451) i.e. a mutation in the ATP binding site in the C-terminal domain, third was a plasmid with 54 amino acid deletion which removes the autoinhibitory alpha helix (697-712) which is located in C-terminal domain (Δα), and last one was with a point mutation in the autoinhibitory alpha helix region (Y707A).

The Rsk2-Δα and the Rsk2-Y707A are considered to be constitutively active Rsk2 expressing plasmids i.e. even if MEK or ERK is inhibited; Phospho-Ser 386 is highly expressed whereas for Rsk2-K451A or Rsk2-WT, no phospho-Ser 386 expression is observed.

All these viruses have pGJ-3 as its expression vector backbone along with CMV promoter and EGFP tagged to the N-terminal of Rsk2 (Raina et al, 2020).

These plasmids were tested by transfecting HEK cells and checking for bands specific to Rsk2 and GFP. When the membranes were probed with either Rsk2 (host-Rabbit) or GFP (host-Rabbit) antibodies, specific bands were observed at 109kDa, as an 82kDa Rsk2 is fused with 27kDa GFP (Fig5)

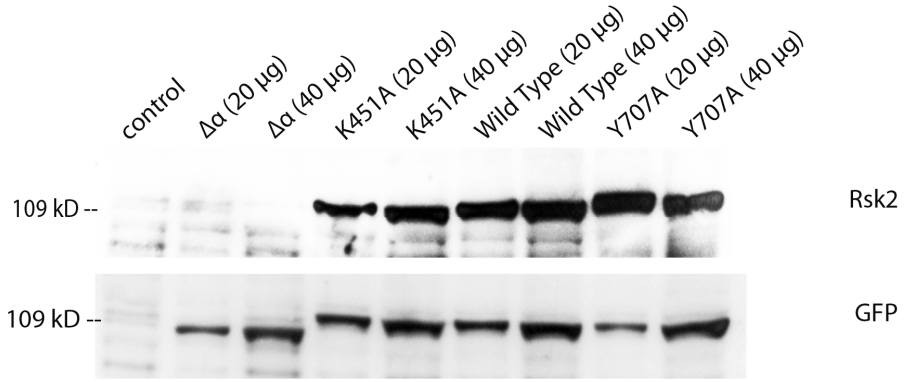


Fig5- HEK cells transfected with DNA plasmids encoding GFP-tagged Rsk2-wt, Δα, K451A and Y707A (20µg and 40µg per well) Bands observed at 109 kDa when probed with Rsk2 and GFP.

Untransfected HEK cells were considered as control. No band was visible for Rsk2-Δα plasmid when probed with Rsk2 antibody because the antibody recognises the C-terminal epitope which has the deletion. However, when probed with GFP antibody, a band could be observed which has lower molecular weight as compared to the others.

3.4. Lentiviral vector production -

After confirming the plasmids are capable of encoding Rsk2, we prepared lentiviral vectors for each of the plasmids by lipofactamine method in HEK293 cells. Along with the expression plasmid vector, VSV-G was used as a pseudotyping vector. No packaging vector was required as all the components were present inside the pGJ-3 vector backbone. The supernatant was harvested 48 hours after transfection. Aliquots of 10µl were made for each viral vector and stored at -80^{o}C (Raina et al, 2021, Psol et al, 2021).

The titre of the viral vectors was tested in HEK293 cells by transducing them and checking the fluorescence after 3 days. Fig6 shows the fluorescence when 5µl of virus added to 200,000 cells.

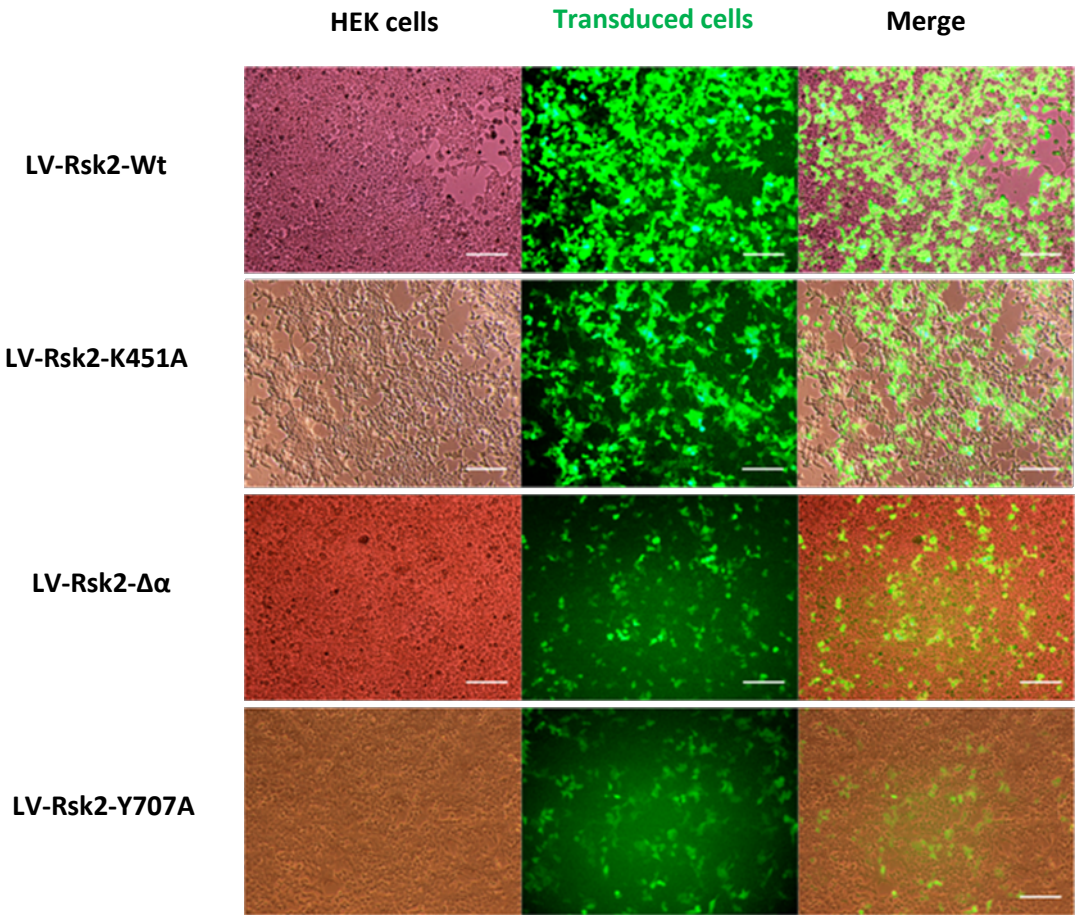


Fig6 - Fluorescence detected when HEK cells were transduced with viral vectors (5µl per 200,000 cells) (Scale bar = 200µm)

3.5. Rsk2 mouse genotyping -

The Rsk2 mice were generated as described in the introduction by Yang et al in 2004. The primers used for genotyping was obtained from Eurofins mwg Operon. Sequence described in Materials and Methods.

(A)

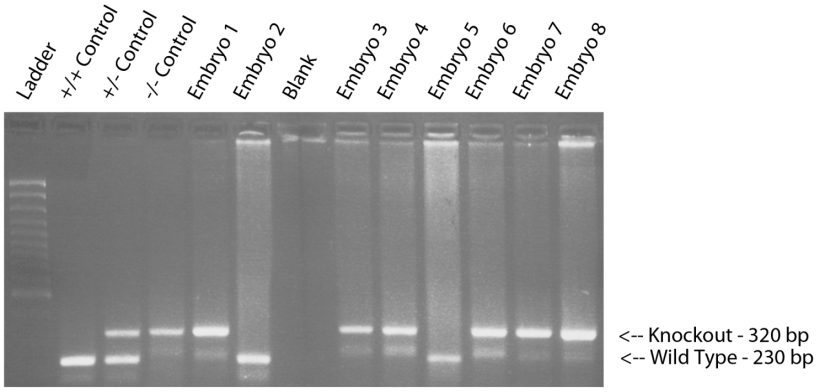
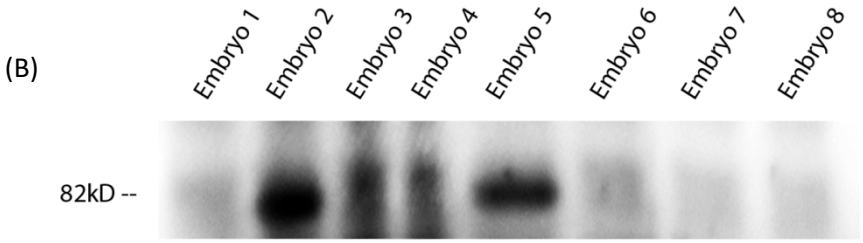


Fig 7 - (A) Genotyping of 8 embryos along with Ladder (1st well) and controls. Homozygous (+/+), Hemizygous (+/-) and knock out (-/-) controls were loaded in the 2nd, 3rd and 4th well respectively.

To confirm the accuracy of the genotyping, spinal cords from the embryos obtained from Rsk2 mouse were taken for Western blotting and heads for genotyping. The spinal cords were lysed and approximately equal amount of protein samples from each spinal cord was loaded for Western blotting. At the same time, DNA purification was done from the heads of the embryos and genotyping was performed using the above mentioned primers. The WT product shows a band at 230 bp whereas the knockout has a band at 320 bp (Fig7-A). For

Western blotting, Rsk2 antibody was used and the expected band would be at 82 kDa (Fig7-B).



(B) 82kDa band specific for Rsk2 seen in 2nd and 5th well, as they are wild types. Others were all knockout and hence, no bands could be observed.

3.6. Rsk2$^{-/-}$ motoneuron culture -

Motoneuron culture was performed after extracting spinal cords from the Rsk2 mouse embryos. Heads were collected in lysis buffer for genotyping. The motoneurons were grown in neurobasal medium supplemented with BDNF. The cultures were maintained at 37^{o}C and in presence of 5% CO_2 for 5 days. Medium was changed every alternate day with fresh medium added to each plate. After 5 days, the motoneurons were fixed with 4% PFA and stained with anti-MAP2 and anti-TAU antibody. The axonal length was measured (Fig8-A) and number of branches were counted (Fig8-B).

(A) (B)

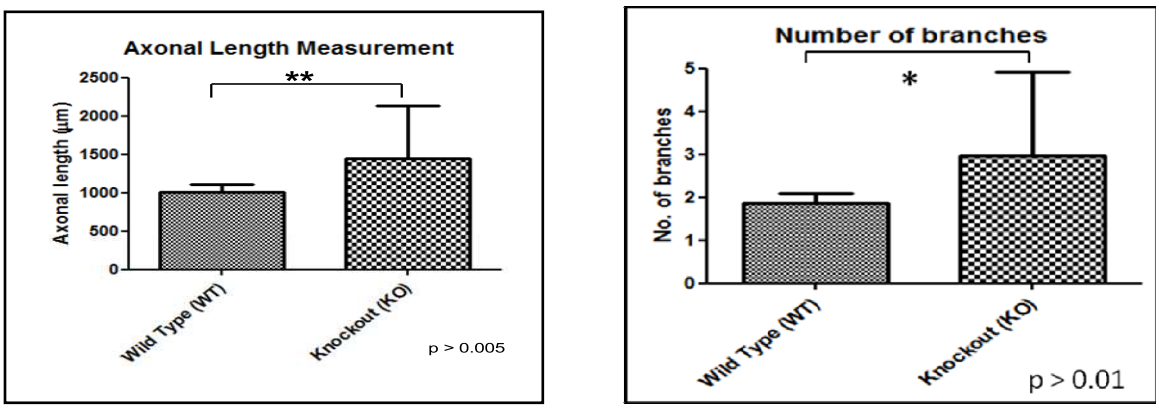


Fig8 - (A) Axons of motoneurons obtained from knockout (-/-) embryos are longer than those seen in wild type (+/+) motoneurons after growing them for 5 days in culture supplemented with BDNF (n=35 for each condition; p>0.005) (B) Knockout motoneurons show higher number of axonal branches as compared to Wild type motoneurons (n=35 for each condition, p>0.01) Both tested with Mann-Whitney test.

These results are in accordance with Dr. Fischer's work, stating that axonal length in Rsk2 deficient motoneurons are longer than the wild type motoneurons *(Fischer et al, 2009).*

Discussion

Motoneurons were cultured in BDNF positive or negative medium for 7 days. These motoneurons were obtained from spinal cords of CD-1 E14 mice embryos and the protocol was similar to that described in *Wiese et al, 2010*. The survival assay performed demonstrated that higher number of motoneurons cultured in BDNF supplemented media survived as compared to the motoneurons cultured in BDNF lacking media (Fig1-A). Other factors like horse serum, B27 and glutamax were provided in similar concentration for both the conditions. These results, as known for a long time, correspond to the fact that BDNF is necessary for growing a better surviving motoneuron culture.

There was no significant difference in the axonal length (Fig1-C) or the number of axonal branches (Fig1-B) between the two conditions. Thus, presence or absence of BDNF majorly affects the survival of motoneurons and not the length of its axons.

When similar experiments were performed in motoneurons obtained from $Rsk2^{-/-}$ mice, it was observed that there is a significant increase in the axonal length in $Rsk2^{-/-}$ mice motoneurons as compared to wild type motoneurons (Fig8-A). An increase was also observed in number of axonal branches in $Rsk2^{-/-}$ motoneurons as compared to wild type ones (Fig8-B). The cultured motoneurons in this case were maintained for 5 days in BDNF positive media. These results were in accordance with the results Dr. Fischer observed. I would like to repeat these experiments again after culturing the motoneurons in BDNF positive media for 7 days. I would also want to compare the survival of $Rsk2^{-/-}$ motoneurons with the wild type motoneurons. However, Dr. Fischer observed no difference in survival of

motoneuron when neurotrophic factors were added and there was higher survival in $Rsk2^{-/-}$ motoneurons when they were absent *(Fischer et al, 2009)*.

They also observed that when $Rsk2^{-/-}$ motoneurons were transduced with constitutively active Rsk2 lentiviral vectors (LV-Rsk2-Δα and LV-Rsk2-Y707A), they showed reduced axonal elongation as compared to the $Rsk2^{-/-}$ motoneurons transduced with Wild type Rsk2 lentiviral vector (LV-Rsk2-Wt). Rsk2 specific bands were observed when the plasmids containing these constructs were probed with Rsk2 antibody in Western blotting after 3 days of HEK cells transfection (Fig5). Later, these lentiviral vectors were prepared to test their effect on $Rsk2^{-/-}$; $Smn^{-/-}$; $SMN2^{tg/tg}$ motoneurons. Their transduction efficiency was checked in HEK cells and the titre of LV-Rsk2-Wt and LV-Rsk2-K451A was found to be approximately 10^9 TU/ml whereas LV-Rsk2-Δα and LV-Rsk2-Y707A was found to be 10^7 TU/ml (Fig6).

It would be interesting to see the effect of Rsk2 in $Smn^{-/-}$; $SMN2^{tg/tg}$ motoneurons. It was shown that the knockout $Smn^{-/-}$; $SMN2^{tg/tg}$ motoneurons have shorter axons as compared to the wild type ones. Smn protein along with its binding partner hnRNP-R aids the transport of β-actin mRNA from the nucleus to the growth cones. Thus, absence of Smn protein leads to reduced transport of mRNA to the growth cones, which is important for axonal growth and guidance *(Rossoll et al, 2007).*

On the other hand, Rsk2 is said to be involved in various cellular functions, one of which is to activate PDK1, which in turn activates Akt which is recruited to the membrane by PIP3. Akt activates mTOR via phosphorylation which is further essential in phosphorylating 4E-BP. 4E-BP, when hypophosphorylated, is said to inhibit local translation initiation in vivo and in

vitro by preventing the assembly of eIF4F complex which is responsible for ribosome recruitment to the mRNA thereby stimulating translation initiation. Rsk2 is also said to play some sort of feedback inhibition mechanism in MAPK signalling pathway. Moreover ERK, an activator of Rsk2, on the other hand can activate mTOR directly as well *(Costa-Mattioli et al, 2009; Wells D, 2006).* Thus, it is clear that Rsk2 plays an important role in the local translation process.

Another paper supporting the role of RSKs in translation initiation was published in 2007 by Roux et al. They provide evidence that RSKs, which are downstream of Ras/ERK signalling pathway, are capable of phosphorylating Ribosomal protein (rp) S6 at $Ser^{235/236}$. This protein is a component of 40S ribosomal subunit required for translation. Interestingly, this phosphorylation of rpS6 via ERK-RSK signalling pathway is said to be independent of mTOR signalling pathway. Phosphorylation of rpS6 at $Ser^{235/236}$ site by RSKs was found to regulate the affinity of rpS6 for the 7-methylguanosine cap complex, indicating that RSK signalling contributes to the assembly of translation initiation complex *(Roux et al, 2007)*.

Rsk2 genotyping was performed with the set of primers mentioned above (Fig7-A). To confirm that there is no Rsk2 expressed in the knockout embryos, Western blotting was done. The results of genotyping perfectly matched the results of the Western, showing no band in the knockout well. But, an 82kDa band could be seen in the well, where proteins obtained from Wild type embryos were loaded (Fig7-B). This not only confirms the genotyping, but also the Rsk2 antibody used for these experiments is specific to Rsk2 and not to other members of the $p90^{rsk}$ family even though they are homologous to each other.

According to paper published by Dugani et al in 2010, there is decreased level of neurogenesis in cultured cortical precursor cells in absence of Rsk2. However, these set of experiments were performed in precursor cells obtained from CD-1 E12 mice embryos and knockdown of Rsk2 was done with Sh-RNA technique (60-80% knockdown of Rsk2). I would like to perform these set of experiment again using $Rsk2^{-/-}$ mice precursor cells where Rsk2 is 100% absent. Therefore, the protocol for neuronal and astrocyte differentiation was standardised using precursor cells obtained from dorsal forebrain of CD-1 E12 mice embryos. Staining was done and differentiated neurons (Fig4-A) and astrocytes (Fig4-B) were observed. However, staining with precursor marker nestin or proliferation marker Ki67 before plating them for differentiation could give a clear idea on the percentage of neurogenesis in presence and absence of Rsk2 (to be performed).

Summary

Cultured motoneurons from E14 CD-1 mice embryos survived longer when the media was supplemented with BDNF. Moreover, no significant difference was observed in axonal length or number of axonal branches in cultured motoneurons in presence or absence of BDNF.

However, cultured motoneurons from $Rsk2^{-/-}$ mice embryos showed longer axons and more branching as compared to their wild type counterparts. This result is in accordance with the results observed by Dr. Fischer.

Culturing of neuronal stem cells and differentiating them into neurons and astrocytes was successful and was confirmed by staining with Tuj1 and GFAP antibodies. The plasmids containing constitutively active Rsk2 and wild type Rsk2 were tested with a specific Rsk2 antibody. Lentiviral vectors generated from these plasmids showed high titre values when transduced in HEK cells.

References

1) Anjum R and Blenis J (2008) The RSK family of kinases: emerging roles in cellular signalling. *Nature Reviews - Molecular Cell Biology* **9**.

2) Bosboom W, Vrancken A, van der Berg L, Wokke J and Iannaccone S (2009) Drug treatment for spinal muscular atrophy type I. *Cochrane Database of Systematic Reviews* **4**.

3) Burghes A and Beattie C (2009) Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? *Nature reviews neuroscience* **10**.

4) Colombi I, Mahajani S, Frega M, Gasparini L and Chiappalone M (2013). Effects of antiepileptic drugs on hippocampal neurons coupled to micro-electrode arrays. Frontiers in neuroengineering 6, 10.

5) Costa-Mattioli M, Sossin W, Klann E, and Sonenberg N (2009) Translational Control of Long-Lasting Synaptic Plasticity and Memory. *Neuron* **61.**

6) Dugani C, Paquin A, Kaplan D and Miller F (2010) Coffin–Lowry syndrome: A role for RSK2 in mammalian neurogenesis. *Developmental Biology* **347**.

7) Fischer M, Pereira P, Holtmann B, Simon C, Hanauer A, Heisenberg M and Sendtner M (2009) P90 Ribosomal s6 kinase 2 negatively regulates axon growth in motoneurons. *Molecular and Cellular Neuroscience* **42**.

8) Frodin M and Gammeltoft S (1999) Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Molecular and Cellular Endocrinology* **151**.

9) Giacomini C, Mahajani S, Ruffilli R, Marotta M and Gasparini L (2016). Lamin B1 protein is required for dendrite development in primary mouse cortical neurons. Molecular biology of the cell 27:35-47.

10) Glinka M, Herrmann T, Funk N, Havlicek S, Rossoll W, Winkler C and Sendtner M (2010) The heterogeneous nuclear ribonucleoprotein-R is necessary for axonal beta-actin mRNA translocation in spinal motor neurons. *Human Molecular Genetics* **19**(10).

11) Hahnen E, Schonling J, Rudnik-Schoneborn S, Zerres K and Wirth B (1996) Hybrid survival motor neuron genes in patients with autosomal recessive spinal muscular atrophy: New insights into molecular mechanisms responsible for the disease. *American Journal of Human Genetics* **59**.

12) Hanauer A Alembik Y, Gilgenkrantz S, Mujica P, Nivelon-Chevallier A, Pembrey M, Young I, Mandel J (1988) Probable localisation of the Coffin-Lowry locus in Xp22.2-p22.1 by multipoint linkage analysis. *American Journal of Medical Genetics* **30**(1-2).

13) Hanauer A and Young I (2002) Coffin-Lowry syndrome: clinical and molecular features. *Journal of Medicine Genetics* **39**.

14) Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M, Paslier D, Frezal J, Cohen D, Weissenbach J, Munnich A and Melki J (1995) Identification and characterization of a spinal muscular atrophy - determining gene. *Cell* **80**.

15) Lorson C, Rindt H and Shababi M (2010) Spinal muscular atrophy: mechanisms and therapeutic strategies. *Human Molecular Genetics* **19**.

16) Lunn M and Wang C (2008) Spinal Muscular Atrophy. *The Lancet* **371**.

17) Mahajani S, Baehr M and Kuegler S (2021). Patterning inconsistencies restrict the true potential of dopaminergic neurons derived from human induced pluripotent stem cells. Neural regeneration research 16:692.

18) Mahajani S, Chavan S, Mahajani S and Raman K (2010). Biodiesel production from algae exposed to stressful conditions. Journal of Pure and Applied Microbiology 4: 339-342.

19) Mahajani S, Giacomini C, Marinaro F, Tonelli D, Contestabile A and Gasparini L (2017). Lamin B1 levels modulate differentiation into neurons during embryonic corticogenesis. Scientific reports 7:1-11.

20) Mahajani S, Raina A, Fokken C, Kuegler S and Baehr M (2019). Homogeneous generation of dopaminergic neurons from multiple hiPSC lines by transient expression of transcription factors. Cell death & disease 10: 1-15.

21) Marotta R, Catelani T, Pesce M, Giacomini C, Mahajani S and Gasparini L (2016). Role of Lamin B1 in structuring the cell nucleaus in eukaryotic cells. European Microscopy Congress 1011-1012.

22) Montes J, Gordon A, Pandya S, De Vivo D and Kaufmann P (2009) Clinical outcome measures in Spinal Muscular Atrophy. *Journal of Child Neurology* **24**.

23) Ogino S and Wilson R (2002) Genetic testing and risk assessment for spinal muscular atrophy (SMA) *Human Genetics* **111**.

24) Pan H, Oliveria B, Saher G, Dere E, ….., Luehder F and Ehrenreich H (2019). Uncoupling the widespread occurrence of anti-NMDAR1 autoantibodies from neuropsychiatric disease in a novel autoimmune model. Molecular psychiatry 24: 1489-1501.

25) Pietro C, Brusco A, Brussino A, Giorgio E, Antonarakis S, … Mahajani S, Giacomini C and Gasparini L (2015). Clinical, neuroradiological and molecular investigation of Adult-onset Autosomal Dominant LeukoDystrophy (ADLD): dissection of Lamin B1-mediated pathophysiological mechanisms in cellular and mouse models. XIII Scientific Convention 39-40.

26) Poteet-Smith C, Smith J, Lannigan D, Freed T and Sturgill T (1999) Generation of Constitutively Active p90 Ribosomal S6 Kinase in Vivo. *The Journal of Biological Chemistry* **274**(32).

27) Psol M, Darvas S, Leite K, Mahajani SU, Baehr M and Kuegler S (2021). Dementia with Lewy bodies – associated β-synuclein mutations V70M and P123H cause mutation-specific neuropathological lesions. Human Molecular Genetics 30, 247-264.

28) Raina A, Leite K, Guerin S, Mahajani SU, Chakrabarti K, …. and Kuegler S (2021). Dopamine promotes the neurodegenerative potential of β-synuclein. Journal of Neurochemistry 156: 674-691.

29) Raina A, Mahajani S, Baehr M and Kuegler S (2020). Neuronal trans-differentiation by transcription factors Ascl1 and Nurr1:induction of a dopaminergic neurotransmitter phenotype in cortical GABAergic neurons. Molecular neurobiology 57:249-260.

Rogers M, Rush R and Sendtner M (2010) Isolation and enrichment of embryonic mouse motoneurons from the lumbar spinal cord of individual mouse embryos. *Nature protocols* **5**(1).

30) Rossoll W, Jablonka S, Andreassi C, Kröning A, Karle K, Monani U, and Sendtner M (2003) Smn, the spinal muscular atrophy–determining gene product, modulates axon growth and localization of β-actin mRNA in growth cones of motoneurons. The Journal of *Cell Biology* **163**(4).

31) Roux P, Shahbazian D, Vu H, Holz M, Cohen M, Taunton J, Sonenberg N and Blenis J (2007) RAS/ERK signalling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates Cap-dependent translation. *The Journal of Biological Chemistry* **282**(19).

32) Sumner C (2006) Therapeutics development for Spinal Muscular Atrophy. *NeuroRx:The Journal of the American Society for Experimental NeuroTherapeutics* **3**.

33) Vitali T, Sossi V, Tiziano F, Zappata S, Giuli A, Petsotas M, Neri G and Brahe C (1999) Detection of Survival Motor Neuron (*SMN*) genes by FISH: further evidence for a role for *SMN2* in the modulation of disease severity in SMA patients. *Human Molecular Genetics* **8**.

34) Wakhloo D, Mahajani S, Tembe P and Raut A (2013). Lactobacillus plantarum shows more probiotic potential than S. cremoris. Journal of Pure and Applied Microbiology 7: 565-570.

35) Wakhloo D, Scharkowski F, Curto Y, … and Ehrenreich H (2020). Functional hypoxia drives neuroplasticity and neurogenesis via brain erythropoietin. Nature communication 11:1-12.

36) Wells D (2006) RNA-Binding Proteins: A Lesson in Repression. *The Journal of Neuroscience* **26**(27).

37) Wiese S, Herrmann T, Drepper C, Jablonka S, Funk N, Klausmeyer A, Wirth B, Herz M, Wetter A, Hahnen M, Rudnik-Schonenorn S, Wienker T and Zerres K (1999) Quantitative analysis of survival motor neuron copies: Identification of subtle *SMN1*mutations in patients with spinal muscular atrophy, genotype-phenotype correlation, and implications for genetic counselling. *The American Journal of Human Genetics* **64**.

38) Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka H, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes T, Hanauer A and Karsenty G (2004) ATF4 Is a Substrate of RSK2 and an Essential Regulator of Osteoblast Biology: Implication for Coffin-Lowry

Syndrome.*Cell* **117**.

39) Zeniou M, Ding T, Trivier E and Hanauer A (2002) Expression analysis of RSK gene family members: the RSK2 gene, mutated in Coffin–Lowry syndrome, is prominently expressed in brain structures essential for cognitive function and learning. *Human MolecularGenetics* **11**(23).

40) Zeniou-Meyer M, Liu Y, Begle A, Olanich M, Hanauer A, Becherer U, Rettig J, Bader M and Vitale N (2008) The Coffin–Lowry syndrome-associated protein RSK2 is implicated in calcium-regulated exocytosis through the regulation of PLD1. *PNAS* **105**(24).